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STUDIES OF GENETIC VARIATION IN THE AIDS VIRUS: RELEVANCE TO DISEASE PATHOGENESIS, ANTI-VIRAL THERAPY, AND VACCINE DEVELOPMENT

ANNUAL REPORT

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MARCH 15, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7038

University of Alabama at Birmingham
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Birmingham, Alabama 35294

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Alabama at Birmingham 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 1900 University Boulevard, THT 513 Birmingham, Alabama 35294	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-87-C-7038
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 623105 / PROJECT NO. 3M2-623105H29 / TASK NO. AD / WORK UNIT ACCESSION NO. 045

11. TITLE (Include Security Classification)
(U) Studies of Genetic Variation in the AIDS Virus: Relevance to Disease Pathogenesis, Anti-Viral Therapy, and Vaccine Development

12. PERSONAL AUTHOR(S)
George M. Shaw

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 12 15/87 TO 12/14/88	1989 March 15	

16. SUPPLEMENTARY NOTATION

Keywords)

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	03		SRA 1; AIDS; HTLV-III; Virology; HIX; Restriction maps; Genetic Maps; (KT)
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Genetic and biologic studies of Human Immunodeficiency Virus Type 1 (HIV-1) aimed at developing an understanding of disease pathogenesis, viral evolution, and methods to treat and prevent infection have expanded to include study of the closely related human and simian immunodeficiency viruses. In our DoD funded work, we are pursuing the contract study aims by molecular analyses of HIV-1 and HIV-2, with focus on four primary aims i) nature and extent of genetic variation, ii) mechanism and rate of viral variation, iii) influence of host immune response on variation, and iv) effect of genetic variation on viral antigenicity, immunogenecity, and biology. In this annual report, we summarize results and conclusions obtained in the past year (year 02) in the areas of HIV-1 variation *in vivo* and genetic and biologic analysis of HIV-2. In these studies, we have demonstrated for the first time that HIV-1 exists as a quasispecies, that genetic variation of HIV-1 develops in parallel with common viral forms persisting over time, and that changes in nucleotide and amino acid sequences accumulate in the envelope immunodominant loop region. In addition, recent clinical and seroepidemiological studies in West Africa indicate that HIV-2 is widespread and associated with immunodeficiency states of variable degree. In this study, a T-lymphotropic human retrovirus, HIV-2/ST, representing the first *bona fide* isolate of HIV-2 from Senegal was molecularly cloned and characterized in regard to its biologic and structural properties and its genetic relatedness to known primate retroviruses. Molecular hybridization and restriction enzyme analysis demonstrated HIV-2/ST to be more related to HIV-2/ROD than to other human or primate retroviruses. Cultures of HIV-2/ST showed genotypic polymorphism, and single-cell derived biological clones of the virus exhibited different-sized transmembrane envelope glycoproteins of 30 and 42 kilodaltons. Unlike other immunodeficiency viruses, HIV-2/ST did not cause cell death or induce cell fusion in PBL or in any of four T4 (CD4) positive cell lines tested. Although HIV-2 entered cells by a CD4 dependent mechanism and replicated actively, cell-free and cell to cell transmission of HIV-2/ST was markedly retarded at the level of cell entry. These findings support the conclusion that immunodeficiency viruses prevalent in human populations in West Africa are members of the HIV-2 virus group, not SIV (simian immunodeficiency virus), and that certain naturally-occurring HIV-2 strains have attenuated virulence. Finally, we show that a novel open reading frame (x) that is present in HIV-2 and SIV, but not HIV-1, encodes an immunogenic protein the function of which is under study. The results of these year 02 studies have led to important progress regarding the goals of this contract and will serve as the basis for future studies.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

HIV-1 Variation *In Vivo*

In year 01 of this contract work, it was shown that sequential isolates of HIV-1 exemplified progressive, cumulative changes in their nucleotide and amino acid sequences as a result of rapid parallel evolution of distinct viral strains *in vivo* (1). To better understand the nature of genetic and antigenic variation of HIV-1 in humans, virus was sequentially isolated and characterized from a previously reported lab worker (LW) infected by the H9/HTLV-IIIb strain of virus. Between 9/4/85 and 5/18/88, 7 HIV-1 isolates were established in 3 independent laboratories from the peripheral blood mononuclear cells (PBMC) of LW by co-cultivation with PBMC of normal donors. Southern blot hybridization patterns of all 7 LW isolates were indistinguishable from each other and from a component of the H9/HTLV-IIIb isolate after digestion with 7 restriction endonucleases (SstI, PvuII, BglII, HindIII, EcoRI, CvnI, KpnI). Recombinant λ phage libraries from LW virus isolates from 9/4/85 (λOF001), 11/30/87 (λFD651), and 5/18/88 (λLW5A) were prepared and 10, 8, and 10 full-length HIV-1 clones, respectively, were analyzed. All 28 clones were identical to each other and to the BH10 clone of H9/HTLV-IIIb in 7 of 7 restriction enzyme patterns, except for 2 clones from the LW5A isolate that differed in single PvuII polymorphisms. The nucleotide and deduced amino acid sequences of the viral envelope gp120 immunodominant loop region (aa302-337) of 3 λOF001 clones, 3 λFD651 clones, and 8 λLW5A clones were determined and compared to a molecular proviral clone BH10 derived from the H9/HTLV-IIIb isolate, as follows:

λBH10 C T R P N N N T R K S I R I Q R G P G R A F V T I G K I G N M R Q A H C

λOF001 - - - - - - - - - - **R** -

λFD651 - - - - - - - - **G** - **R** - - - - - - - - - **T** - - - - - - - - - - - - - -

λLW5A - - **K** - - - - **G** - **R** - - - - - - - - - **T** - - - - - - - - - - - - - -

Clones from within each of the 3 LW isolates were identical to each other but between isolates they showed cumulative nucleotide point mutations that resulted in the amino acid changes shown. The envelope genes from λOF001, λFD651, λLW5A, and λBH10 clones were

substituted into the HIV-1 proviral expression vector pHXB-2d and transfection-derived virus strains generated for analysis of antigenic and biologic properties. These studies demonstrate that cumulative changes develop in immunologically relevant regions of the HIV-1 envelope and will provide insight into the nature and significance of host-viral interactions mediated by this viral protein. Although our analyses are not yet completed, these approaches are going to enable us to define molecularly the interaction between host immune system and viral amino acid sequences.

In parallel with these studies in which virus has been sequentially isolated, cloned, sequenced, and transfected, we have also developed PCR (polymerase chain reaction) amplification as a method to clone and sequence HIV-1 genomes directly from uncultured patient tissues, avoiding virus culture. This is important because potential artifacts could be introduced during the culture process, especially the selective gain or loss of biologically important viral species. The following five sets of oligonucleotide primer pairs have been prepared and used to amplify HIV-1 and control DNA sequences:

PRIMER 589/590
P589 5' CTCGAGGTGTCAACTCAACTGCTGTTAAATGGCAGT 3'
P590 5' GGATCCTTGTTAACAGCAGCCCTGTAATATTTGATG 3'
PROBE 5' CTAGCAGAAGAAGAGGTAGTAATTAGATCT 3'

PRIMER 3/4 (HIV-1 pol)
P3 5' TTCTGGGAAGTTCAATTAGGAATACC 3'
P4 5' CCTACATACAAATCATCCATGTATTC 3'
PROBE 5' ATGAGACACCAAGGGATTAGATATCAGTACA 3'

PRIMER 5/6 (HIV-1 pol)
P5 5' AAATTAGCAGGAAGATGGCCAGTA 3'
P6 5' CTACTCCTTGACTTTGGGGATTGT 3'
PROBE 5' CCACCAACAGGCGGCCTTAACCGCAGCACT 3'

PRIMER RS79/RS80 (β-globin)
RS79 5'GTATCATGCCTCTTTGCACCATTC 3'
RS80 5'GCAGAATGGTAGCTGGATTGTAGC 3'
PROBE 5'CTGGGTTAAGGCAATAGCAATATTTCTGCA 3'

PRIMER A26/A27 (HLA DQα)
A26 5'CTCTTGTGGTGTAAACTTGTACCAG 3'
A27 5'CTCATTGGTAGCAGCGGTAGAGTTG 3'

Using these sets of HIV-1 and control (β globins and HLA DQα) primers we have been

able to develop a quantitative assay for HIV-1 sequences in infected patient tissues and to molecularly clone HIV-1 sequences from uncultured patient tissues into M13 phage for sequence analysis. This will allow, for the first time, direct analysis of HIV-1 genetic variation and heterogeneity *in vivo*. The results of these studies using uncultured patient tissues show that HIV-1 *in vivo* exists as a quasispecies and that common and variant viral forms evolve and persist over time. In studies that are continuing into year 03, we are using a combination of PCR and classical phage cloning techniques to fully characterize HIV variation *in vivo* and to study its biologic relevance.

Isolation and Genetic and Biologic Analysis of HIV-2

Although human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of epidemic AIDS in Central Africa, Europe, the United States, and most countries worldwide (2), many cases of AIDS in West Africa have been attributed to a different but related retrovirus, human immunodeficiency virus type 2 (HIV-2) (3, 4). Although it is clear that HIV-2 in many instances causes fatal immunodeficiency (4), recent studies (5, 6) have suggested that many West African individuals as a group, when infected with HIV-2, may have less severe immunodeficiency than individuals infected with HIV-1. These studies involved widespread serologic testing of West African populations by immunoblot and radioimmunoprecipitation (RIP) assays with viral-specific target antigens derived from cultures of HTLV-4/PK82 (7), a virus strain now known to represent a simian retrovirus termed SIV_{MAC} (simian immunodeficiency virus of macaques) (8-11). In our studies, we have characterized an HIV-2 related virus (HIV-2/ST) from a healthy prostitute in Senegal, West Africa which represents the first independent virus isolation from this region. The biologic and genetic properties of this virus distinguish it from other known immunodeficiency-associated retroviruses, and raise the possibility that such strains of HIV-2 may be responsible for altered disease presentations in naturally infected human populations.

HIV-2/ST was isolated from peripheral blood mononuclear cells (PBMC) of patient ST

as described (9). Virus was transmitted to four immortalized T-cell lines (Hut78, H9, SupT1, and CEMx174) using repeated PEG (polyethylene glycol) precipitations of primary culture supernatants in an attempt to isolate and amplify viruses with potentially attenuated virulence. The DNA genome of HIV-2/ST in these cultures hybridized at low and high stringency to the prototype HIV-2/ROD probe (Fig. 1A, lanes 5 and 6) whereas SIV_{MAC} DNA hybridized to the HIV-2/ROD probe only under conditions of low stringency (Fig. 1A, lanes 3). Conversely, HIV-2/ROD and HIV-2/ST DNA hybridized with equal intensity to SIV_{MAC} probe, but only at low stringency, and a full-length HTLV-1 probe gave no hybridization. Two different HIV-2/ST producing cell lines derived from the transmission of cell-free virus from primary ST lymphocyte co-cultures exhibited very similar proviral restriction enzyme cleavage patterns but were distinguishable by polymorphic Xba I and Bam HI sites (Fig. 1B). A recombinant DNA lambda phage library prepared from one of these cell lines, Hut 78/B12, which represented a biologically cloned high producer line, yielded three full-length proviral DNA clones that were evaluated by detailed restriction analyses (Fig. 1C). Comparative restriction mapping confirmed the genetic similarity between HIV-2/ST and HIV-2/ROD (10 of 25 restriction sites in common) and the dissimilarity between HIV-2/ST and SIV_{MAC} (2 of 36 restriction sites in common). Furthermore, one of these proviral DNA clones (lambda JSP4-27), when transfected into T-lymphocyte cell lines, produced virus that was replication-competent and infectious and whose biological properties were identical to the parental virus isolate. Thus, HIV-2/ST is genotypically more related to HIV-2/ROD than to other primate viruses, and within the HIV-2/ST isolate there exist polymorphic genotypes some of which are fully replication competent.

The structural, antigenic, and functional characteristics of HIV-2/ST proteins were evaluated by immunoblot and RIP analyses (Fig. 2). The putative major *gag* structural protein and precursor (p26/pr55) of HIV-2/ST were comparable in size to p26/pr55 of HIV-2/ROD, p27/pr55 of SIV_{MAC}, and p24/pr53 of HIV-1. Polymerase and endonuclease proteins of HIV-2/ST were tentatively identified as p64 and p34 proteins with equivalent counterparts in HIV-2/ROD, SIV_{MAC}, and HIV-1. The *env* proteins of HIV-2/ST revealed a 140 kD putative

extracellular protein similar to that in HIV-2/ROD. However, in different subcultures of HIV-2/ST that were derived by single cell cloning of the original SupT1/LK001 cell line, the putative transmembrane proteins (TMP) were either 42 kD or 30 KD in size. Only smaller-sized transmembrane proteins were seen in HIV-2/ROD (36 kD) and SIV_{MAC} (32 kD) cultures. The HIV-2/ST viruses with large and small transmembrane proteins could be transmitted cell free, were replication competent, and retained the same size of TMP throughout passage in the same cell targets (SupT1 and CEMx174). This suggests that the observed differences in TMP sizes were due to nucleotide sequence differences in the expressed mRNAs, not to post-transcriptional modifications such as altered glycosylation patterns. Consistent with this suggestion is the finding of translational stop codons in HIV-2/ROD and SIV_{MAC} viral envelope genes which result in the expression of truncated TMPs (12). Antigenically, HIV-2/ST and HIV-2/ROD were similar to each other and to SIV_{MAC} in their reactivity with HIV-2 antisera and were cross-reactive with HIV-1 only in the major *gag* structural proteins (p24-p27). This was confirmed by bi-directional immunoblotting in which both HIV-1 and HIV-2 specific human antisera were used to probe HIV-1, HIV-2/ROD, and HIV-2/ST proteins (Fig. 2A). By RIP, we found *env* precursor proteins of approximately 180 or 170 kD in the different HIV-2/ST subcultures (reflecting the different sizes of their respective transmembrane proteins) and mature extracellular envelope proteins of 140 kD (Fig. 2B). By RIP, we also found that, like HIV-1, the HIV-2/ST external *env* glycoprotein bound directly to an epitope on CD4 recognized by OKT4A (Fig. 2C), but not by OKT4 (13). Other experiments (14) with fluorescence activated cell sorting analysis showed that infection of CD4 bearing cells by HIV-2/ST virus down-modulated the expression of CD4(T4) but not CD8(T8), again analogous with HIV-1 infection (15).

Because of the evidence suggesting that certain West African human populations infected with HIV-2 related viruses may have less severe immunodeficiency than do individuals infected with HIV-1, or some isolates of HIV-2 including HIV-2/ROD (5, 6), we evaluated HIV-2/ST virus for cytopathic and cell killing properties *in vitro*. We first studied the induction

of syncytia by HIV-2/ST infected cells co-cultured with a panel of different $CD4^+$ cells. As shown in Fig. 3, both HIV-1/IIIb and HIV-2/ROD, but not HIV-2/ST, produced large syncytia when virally-infected cells were co-cultured with uninfected $CD4^+$ cells. Hela-T4, H9, and Hut 78 indicator cells gave identical results. Syncytia-induction was inhibited by the monoclonal antibody Leu3a and no syncytia were formed by any HIV-1 or HIV-2 infected cells when co-cultured with $CD4^-$ cells. Cytopathicity was also assessed by direct determination of cell killing. HIV-2/ROD and two different isolates of HIV-1 (IIIb and BC) caused marked cell killing of SupT1 cells (Fig. 4, A and B), PHA-stimulated normal donor lymphocytes (Fig. 4, C and D), as well as Hut78 and H9 cells (not shown). Equivalent amounts of HIV-2/ST showed no detectable cell killing activity against the same cell targets. Even 10- and 100-fold more HIV-2/ST virus than three other HIV-1 isolates (WMJ, RH, BC) led to only transient depression in cell counts (Fig. 4, E). These cell-killing experiments were repeated three times with identical results, each time with the use of newly concentrated virus stocks. Syncytia induction that accompanied virus infection in these experiments was also scored and was dramatic in size and number in cultures infected by HIV-2/ROD and the four HIV-1 isolates but absent in cultures infected with HIV-2/ST. The virus stocks used for these experiments were obtained from the original uncloned HIV-2/ST infected line SupT1/LK001 and were infectious and replication-competent on the basis of their ability to infect, replicate in, and reinfect by cell-free passage $CD4^+$ cells. Furthermore, the cell targets shown in Figure 4, and the virus infected cells used for the syncytia induction assays in Figure 3, were comparably infected with HIV-2/ST, HIV-2/ROD, and HIV-1 based on viral-specific immunofluorescence, *in situ* hybridization, and supernatant particulate reverse transcriptase assays.

In previous studies of the infectivity and fusion activity of SIV_{MAC}, it was observed that a cell line (CEMx174) representing a somatic cell hybrid between a T-cell (CEM) and a B-cell (B721.174), but neither parental cell line itself, was exquisitely sensitive to virus infection and syncytia induction (16). We therefore used this hybrid cell line (CEMx174) in cytopathicity assays with HIV-2/ST. CEMx174 cells were highly susceptible to HIV-2/ST infection and were

permissive for permanent, high-titer virus production. Syncytia induction and cell-killing assays showed a modest cytopathic effect but again much less than for prototype strains of HIV-1 and HIV-2. To evaluate the basis for the attenuation of HIV-2/ST, we used *in situ* hybridization to analyze at the single cell level the rate of virus entry and early gene expression after cell-free viral transmission (Table 1). Compared to prototype strains of HIV-1 and HIV-2, HIV-2/ST showed markedly delayed onset of viral RNA production in CEMx174 (Table 1) as well as in SupT1 and Hut 78 following exposure to cell free virions. However, once infected, cells expressed equivalent amounts of HIV-2/ST RNA as compared with HIV-2/ROD and HIV-1/IIIb, and produced equivalent amounts of mature virus as assessed by RT activity and viral structural proteins. This, and the fact that HIV-2/ST is less fusogenic, suggests that the defect in HIV-2/ST is at the level of virus entry, not expression.

Previous studies (3, 4, 12) have shown that viruses of the HIV-2 group may cause severe and fatal immunodeficiency. Viruses cultured from these diseased patients were readily isolated by standard lymphocyte co-culture techniques and showed peaks of supernatant RT activity and cytopathic effects within the first 2 weeks of culture, similar to HIV-1. We had considerable difficulty in establishing virus isolates from healthy Senegalese subjects. Out of four patients originally studied (9), RT activity in lymphocyte co-culture supernatants was detected in three, but in only one case was a virus (HIV-2/ST) successfully amplified and transmitted to permanently producing immortalized T-cell lines. Taken together with the cytopathicity and infectivity data, these results suggest that certain strains of HIV-2 may be less virulent than others and in turn be associated with a less severe or altered expression of immune deficiency. The molecular basis for the attenuated virulence of HIV-2/ST *in vitro* is not known. However, the demonstration that gp120-T4(CD4) binding is followed by down-modulation of cell surface T4(CD4) expression but not cell fusion, and the apparent delay in cell entry and spread after cell-free viral transmission, suggest that changes in the viral envelope important for membrane fusion or other post-binding events are involved. We have also isolated from a single subject on two occasions a strain of HIV-1 that, like HIV-2/ST, was

poorly transmissible and required repeated PEG precipitations of culture supernatants for successful amplification. Mullins and co-workers (17) have recently described a replication defective strain of feline leukemia virus (FeLV) whose variant *env*/LTR region is actually responsible for *enhanced* virulence and they have suggested that biologically important strains of human immunodeficiency viruses could have similarly altered genetic and biologic properties. This report of an attenuated form of HIV-2 is an example of such a naturally-occurring HIV variant.

The significance of different-sized *env* transmembrane glycoproteins in HIV-2/ST is unknown. Biologically-cloned cultures of HIV-2/ST with either large (42 kD) or small (30kD) TMPs were both infectious, replication-competent, and non-cytopathic as were isolates of SIV_{MAC} and HIV-2/ROD having only small TMPs. These data suggest that a full-length (gp 42) TMP is not required for any of these biological functions, although the carboxyterminus of the TMP reading frame, which is highly conserved among HIV-1, HIV-2, and SIV_{MAC}, could potentially encode a separate protein with distinct biologic function.

Considerable controversy has surrounded the question as to what types of human immunodeficiency viruses, besides HIV-1, are present in West Africa. HIV-2/ST was shown by differential nucleic acid hybridization, restriction mapping, and analysis of core proteins to be considerably more related to the prototype HIV-2/ROD virus than to HIV-1 or SIV_{MAC}. There was no evidence in any of the primary or secondary cultures of HIV-2/ST, or in cultures from the other three Senegalese subjects similarly studied, for the presence of an SIV_{MAC}-like virus. Because of the near identity among isolates of HTLV-4 and SIV_{MAC}-251 at the genetic level, the failure to reisolate similarly conserved SIV_{MAC}-like viruses from West Africans, and the general finding of genotypic diversity among independent isolates of HIV-1, HIV-2, and SIV_{MAC}, we conclude that the HIV-2 viruses typified by HIV-2/ROD (2, 3), HIV-2/SBL6669 (18), and HIV-2/ST, along with HIV-1, represent the naturally occurring human immunodeficiency viruses in West Africa. In year 03 of our DoD supported studies, we will perform nucleotide sequence analysis of the HIV-2/ST viral genomes and attempt to define genetic determinants of viral

virulence.

Identification of the X Gene in HIV-2 and SIV_{MAC}

As part of our studies involving the genetic and biologic characterization of HIV-1 and HIV-2 viruses, we have also focused attention on unique features of central region genes that distinguish HIV-1 from HIV-2. Nucleotide sequence comparisons of HIV-1, HIV-2 and SIV_{MAC} have demonstrated an overall highly conserved genomic organization, represented by LTR-gag-pol-vif-central region-env-3'orf-LTR (19-25). HIV-2 and SIV_{MAC} are approximately 70% homologous at the nucleotide sequence level and each is approximately 45% similar to HIV-1 (20, 21). Interestingly, there is an additional open reading frame in the genomes of HIV-2 and SIV_{MAC} that is missing in HIV-1. This open reading frame, termed X, is situated in the central viral region between vif and vpr genes and partially overlaps the vif open reading frame on its 5' end. The deduced amino acid sequence of X predicts a protein of 112 amino acids and 14.5kD calculated molecular weight and is conserved in 94 of 112 amino acid residues between HIV-2 and SIV_{MAC} (Figure 5). In this study we chemically synthesized HIV-2 and SIV_{MAC} X specific oligopeptides, raised heterologous antisera to these peptides in rabbits, and expressed the SIV_{MAC} X open reading frame as TrpE fusion proteins in *E. coli*. With these reagents, we have demonstrated that X encodes a novel retroviral protein uniquely present in HIV-2 and SIV_{MAC} and have provided evidence for its expression and immunogenicity *in vivo*.

Two synthetic peptides encompassing 19 residues from the N-terminus of the deduced X amino acid sequences of HIV-2/ROD and SIV_{MAC}/PK82 (underlined in Figure 5), were synthesized and used to generate rabbit immune sera. The peptides were prepared by solid phase methodology on an Applied Biosystems 430-A peptide synthesizer using a paramethylbenzhydrolamine resin (29). Synthetic peptides were coupled to keyhole limpet hemocyanin (KLH) at the carboxyterminus, after the addition of two glycine spacers and a cysteine residue. New Zealand white rabbits were immunized with 1mg of KLH-coupled peptides emulsified 1:1 in Freunds complete adjuvant, boosted twice at two week intervals with

the same peptides mixed 1:1 with Freunds incomplete adjuvant, and bled six weeks after the first immunization to collect immune sera. A peptide ELISA with non-KLH-coupled oligopeptides as antigen was performed to confirm high titer antipeptide antibodies in these sera (30).

The SIV_{MAC} X orf was also expressed as a fusion protein in *E. coli*. An Xho-II/HindIII fragment containing the entire SIV_{MAC} X open reading frame minus the first six nucleotides (SIV_{MAC} clone PKE 102, ref. 26) was subcloned into the bacterial expression vector pATH (31), immediately adjacent and inframe with the bacterial Trp-E gene (pATH-X, Figure 6a). *E. coli* (HB101) transformed with pATH-X were treated in mid-log phase growth with Indole acrylic acid to induce the trp operon (32). Cell extracts were subjected to polyacrylamide gel electrophoresis, and a predominant protein of approximately 50kD, consistent with the predicted molecular weight of a TrpE/X fusion protein, was identified by Coomassie blue staining of sodium dodecyl sulfate polyacrylamide gels. A second plasmid construct was prepared by deleting an internal NruI/EcoRI fragment in pATH-X, which removed 95% of the TrpE coding sequence (Figure 6a). This plasmid retained only 16 amino acid residues derived from the TrpE gene on the N-terminus and resulted in an expressed X fusion protein of 15kD (delta TrpE/X). Both the TrpE/X fusion protein and the TrpE deleted X fusion protein comprised approximately 20% of the total cellular protein as judged by Coomassie blue staining of gel electrophoresed bacterial lysates. Sera from rabbits immunized with the SIV_{MAC} and HIV-2 X oligopeptides (Figure 6b, panels B and D, respectively) reacted strongly with these lysates on Western blots (TrpE/X lanes 2; delta TrpE/X lanes 3), but no reactivity was detectable with the corresponding pre-immune sera (panels A and C), or with lysates of cultures transformed with the non-recombinant vector (lanes 1). An anti-TrpE antiserum, used for control, detected both the 37kD TrpE protein alone as well as the 50kD TrpE/X fusion protein, but did not recognize the 15kD TrpE deleted X protein (panel E).

In order to identify the putative X protein in SIV_{MAC} and HIV-2 infected cells and cell-free virions, immunoblotting techniques were used to probe viral preparations for reactivity with

the rabbit immune sera. Figure 7a shows the Western blot patterns of viral preparations from cultures infected with HIV-1 (lanes 1), HIV-2 (lanes 2), SIV_{MAC} (lanes 3), as well as mock-infected cells (lanes 4). A polyclonal human anti-HIV-1 serum (RR) and a polyclonal human anti-HIV-2 serum (ST) were used for control to confirm the presence of virus specific antigens. Probing with the X-specific rabbit immune sera (anti-SIV_{MAC}, panel B; anti-HIV-2, panel D) resulted in the identification of a 14 and 12kD viral protein in HIV-2 and SIV_{MAC} viral preparations, respectively, but not in similarly prepared preparations of HIV-1 or virally-uninfected control cultures. Similar results were obtained using whole cell lysates infected with these viruses. No reactivity was detected with the preimmune sera (panels A and C). Interestingly, sera from rabbits immunized with the HIV-2 X peptide recognized only the homologous HIV-2 X protein (panel D), while the SIV_{MAC} X peptide antisera recognized both the HIV-2 and the SIV_{MAC} X protein (panel B). The SIV_{MAC} X protein appeared to be slightly smaller as compared to HIV-2 and was reproducibly present in considerably less amounts. The significance of these differences is presently under study.

In order to determine whether the X protein was actually virion-associated, HIV-2/ST virus was first concentrated by ultracentrifugation and then further purified on a 20% to 60% continuous sucrose gradient prior to Western blot analysis. As shown in Figure 7b, HIV-2 X peptide immune sera (D) detected the identical 14kD X-protein on immunoblots of banded HIV-2 virions. The intensity of this X specific band was equal to slightly greater as compared to cell lysates infected with comparable amounts of HIV-2. A human serum (ST) from a healthy HIV-2 infected individual, and the preimmune rabbit antiserum, failed to detect the X protein in the same antigen preparation. These data were confirmed with a second HIV-2 isolate (HIV-2/ROD).

Identification of the 12-14kD protein as the virally encoded X gene product was further established by competitive adsorption assays (Figure 7c). SIV_{MAC} and HIV-2 X immune sera were adsorbed with nitrocellulose-immobilized lysates of *E. coli* transformed either with the pATH cloning vector or with the pATH-X recombinant plasmid. Adsorption of the SIV_{MAC} X

immune sera with the 50kD TrpE/X fusion protein resulted in a complete loss of antibody reactivity with both HIV-2 (panel B',lane 2) and SIV_{MAC} (panel B', lane 3) viral preparations. Preadsorption of the same sera with *E. coli* lysates transformed with the pATH vector alone gave hybridization signals equal to that in Figure 7a, panel B (not shown). The reactivity of HIV-2 X peptide antisera (D') was only slightly diminished by preadsorption with the SIV_{MAC} TrpE/X fusion protein, as expected, since this antiserum had failed to recognize the heterologous X protein on previous immunoblots (compare Figure 7a, lane 3, panel D). Taken together, these results confirmed the 12-14kD proteins as the HIV-2 and SIV_{MAC} virally encoded X proteins.

To evaluate whether the SIV_{MAC} X protein synthesized in *E. coli* could be of diagnostic value, we examined a limited number of patient and normal control sera for reactivity with this protein (Figure 8). Four sera from healthy West African HIV-2 infected individuals (a-d), four sera from AIDS patients seropositive for HIV-1 (e-h), and two healthy control sera (i-j) were tested side-by-side for reactivity to the 50kD TrpE/X protein (lanes 2), the 15kD TrpE deleted X protein (lanes 3), and lysates of bacteria transformed with the non-recombinant pATH vector (lanes 1). Out of four HIV-2 antibody positive sera tested, two contained antibodies reactive with both the 50kD TrpE/X and the 15kD delta TrpE/X protein (panels a and c). These sera titered to a dilution of 1:50. Four HIV-1 positive sera and two HIV negative control sera did not react with these X specific fusion proteins (panels e-j). Bands corresponding to bacterial proteins other than the X fusion proteins were also observed, particularly with the West African sera. However, these were easily distinguishable from X-specific reactivities by their size and occurrence in the control antigen preparations (lanes 1).

In summary, the data demonstrate that HIV-2 and SIV_{MAC} viruses encode a novel retroviral protein, termed X, which is expressed in virus cultures *in vitro* and in naturally-infected humans, *in vivo*. The molecular size of this protein, 12-14kD, approximates that calculated from the deduced amino acid sequence, although the actual structure and organization of the X gene and its protein, and the mode of X gene transcription in HIV-2 and

SIV_{MAC} viruses, cannot be predicted. Similarly unknown at the present time is the function of the X protein in the life cycle of HIV-2 and SIV_{MAC}. The high degree of sequence conservation suggests a significant function for X in both viruses. The fact that HIV-1 lacks the X gene entirely, yet causes disease in infected individuals, implies that X gene expression is not required for induction of cytopathicity *in vitro* or *in vivo*. A search for X-related protein sequences in the NBRF protein data bank identified several proline-rich regions present in retroviral proteins, adenovirus, papillomavirus, human collagen, and a group of human phosphoproteins to share homology with the carboxyterminus of the X gene. In addition, a probable nuclear antigen of Epstein-Barr virus (34) showed 10 of 14 conserved amino acids with a region of X just 5' of the polyproline tract ($GRG^{H}_{R}G^{P}_{R}GG^{W}_{G}RPG^{P}_{A}P$). The significance of these homologies is under study.

The presence of antibodies specific for X in some HIV-2 infected individuals demonstrates both its immunogenicity and *in vivo* expression. It will be important to determine if X specific reagents will be generally useful in distinguishing between HIV-2 and HIV-1 infection, whether expression of X *in vivo* influences viral pathogenesis and clinical outcome, and whether titers of X specific antibodies will provide prognostically important clinical information. The availability of X specific reagents that we have developed as part of the current DoD contract should facilitate experiments addressing these questions.

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Table 1. Time course of viral infection by HIV-1_{IIIb}, HIV-2_{ROD}, and HIV-2_{ST} assessed by in situ hybridization. The relative amount of viral mRNA production per cell was scored qualitatively from silver grain densities: -, absent, to ++++, too numerous to count. Although there was a marked delay in the initial development and spread of productive viral infection by HIV-2_{ST}, the few cells that became productively infected at early time points expressed equal amounts of viral RNA on a per cell basis compared with prototype HIV-1 and HIV-2

Isolate	Cells expressing viral mRNA (%)	Viral mRNA per cell
	Day 1 after infection	
HIV-1/IIIb	3	++++
HIV-2/ROD	8	++++
HIV-2/ST	0.01	++++
Control	0	-
	Day 3 after infection	
HIV-1/IIIb	35	++++
HIV-2/ROD	45	++++
HIV-2/ST	0.01	++++
Control	0	-
	Day 7 after infection	
HIV-1/IIIb	100	++++
HIV-2/ROD	100	++++
HIV-2/ST	0.5	++++
Control	0	-
	Day 10 after infection	
HIV-1/IIIb	100	++++
HIV-2/ROD	100	++++
HIV-2/ST	10	++++
Control	0	-
	Day 14 after infection	
HIV-1/IIIb	ND	ND
HIV-2/ROD	ND	ND
HIV-2/ST	50	++++
Control	0	-

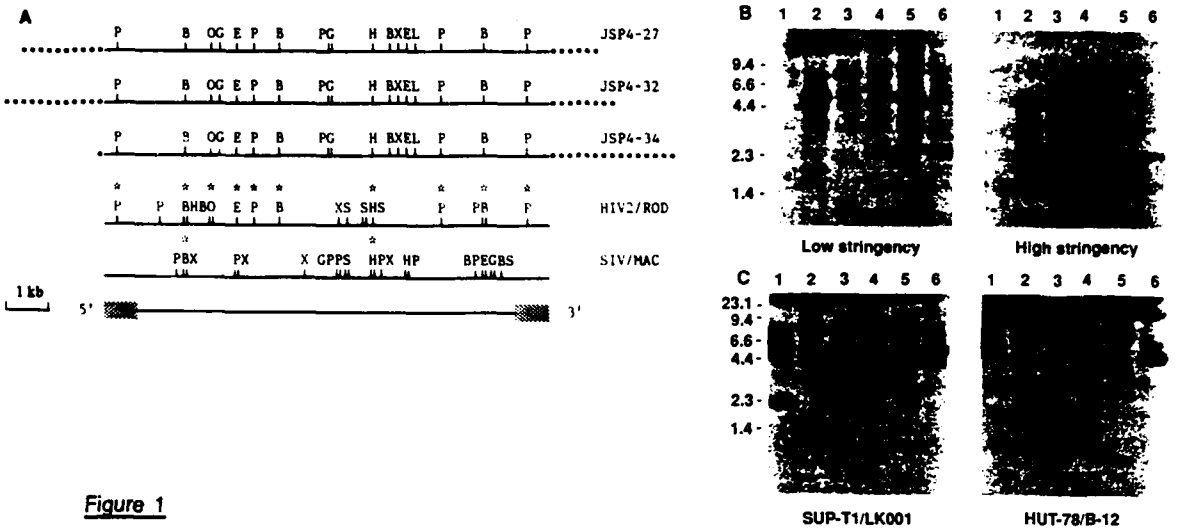

Figure 1

Fig. 1. (A) Genetic characterization of HIV-2/ST by differential nucleic acid hybridization. (B) Restriction enzyme analysis of HIV-2/ST DNA. (C) Comparison of full-length HIV-2/ST, HIV-2/ROD, and SIV_{MAC} proviral DNA clones. (A) Identical nitrocellulose filters containing agarose gel separated Bam HI restriction digests of virally-infected cellular DNA (10ug each) were prepared as described (1): HIV-1/IIIb (lanes 1); HIV-2/ROD (lanes 2); SIV_{MAC}-251 (ref. 19) (lanes 3); normal human lymphocyte DNA (lanes 4); HIV-2/ST (cell line SupT1/LK001) (lanes 5); and HIV-2/ST (cell line Hut78/B12) (lanes 6). Hybridization was to a ^{32}P-labelled 4.3 kb Pst I-Pst I (*pol*-central region-*env* fragment of HIV-2/ROD probe (Fig. 1C) and filters were washed at low (3 x SSC, 0.2% SDS, 55°C) or high (0.1 x SSC, 0.2% SDS, 65°C) stringency. (B) DNA blot-hybridization restriction cleavage analysis with the same probe as in (A) and performed as described (1). SupT1/LK001 and Hut 78/B12 are permanent producing HIV-2/ST infected cell lines, the latter derived by single cell cloning of a primary Hut 78 infected line. Restriction enzymes were Xba I (lanes 1), Xho I (lanes 2), Bam HI (lanes 3), Eco RI (lanes 4), Hinc II (lanes 5), and Kpn I (lanes 6). (C) Restriction maps of three full-length recombinant proviral DNA clones of HIV-2/ST (JSP4-27; JSP4-32; JSP4-34) obtained by Sau3AI (Mbo I) partial digestion of Hut 78/B12 DNA and ligation into J1-lambda phage, as described (21). Restriction enzymes shown are Pst I (P), Bam HI (B), Xho I (O), Bgl II (G), Eco RI (E), Hind III (H), Xba I (X), Sal I (L), and Sst I (S). Asterisks denote sites in HIV-2/ROD or SIV_{MAC} that are also present in the HIV-2/ST clones. Dotted lines denote unique flanking cellular sequences in the HIV-2/ST clones.

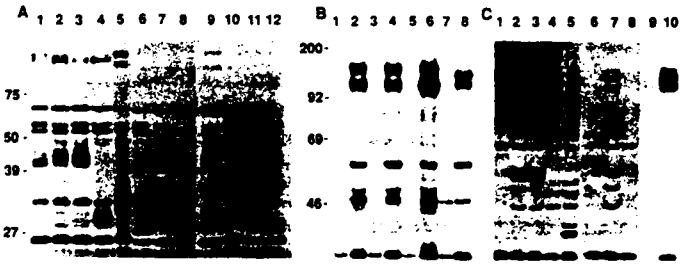

Figure 2

Fig. 2. (A) Western immunoblot and (B and C) RIP analysis of HIV-2/ST, HIV-2/ROD, SIV_{MAC}, and HIV-1/IIIb. (A) Immunoblots were performed using a human (West African) antiserum to HIV-2 (lanes 1-8) and a human antiserum to HIV-1 (lanes 9-12). Bound antibody was detected with peroxidase-conjugated goat antibody to human immunoglobulin. Antigen preparations were viral lysates from HIV-2/ST line SupT1/LK001 (lanes 1, 12); single cell derived clones from SupT1/LK001 designated ST.17 (lane 2), ST.9 (lane 3), ST.24 (lanes 4, 11); HIV-2/ROD (lanes 5, 10); SIV_{MAC} (lanes 6); HIV-1/IIIb (lanes 7, 9); uninfected control cells (lane 8). (B) RIP of HIV-2/ST *env* proteins from cloned cell lines of infected SupT1 cells shown in (A). Cell lysates from [^{35}S]cysteine and [^{35}S]methionine labeled clones were prepared and immunoprecipitated with either normal human serum (lanes 1, 3, 5, 7) or serum from a West African patient with HIV-2 infection (lanes 2, 4, 6, 8). Shown are clones designated ST.9 (lanes 1 and 2), ST.17 (lanes 3 and 4), ST.23 (lanes 5 and 6) that have an *env* precursor molecule of approximately 180 kD and ST.24 (lanes 7 and 8) which has an *env* precursor molecule of 170 kD. The smaller sized *env* precursor (gp170) of ST.24 corresponds to the truncated transmembrane protein (gp30) of the same virus (Fig. 1A). (C) Co-precipitation of HIV-2/ST envelope glycoproteins with CD4. Cell lysates from [^{35}S]cysteine and [^{35}S]methionine-labeled uninfected SupT1 cells (lanes 1-5) and HIV-2/ST infected SupT1/LK001 cells (lanes 6-10) were prepared and immunoprecipitated with OKT3 (lanes 1 and 6), OKT4 (lanes 2 and 7), OKT4A (lanes 3 and 8), normal human serum (lanes 4 and 9), or human serum from a patient with HIV-2 infection (lanes 5 and 10). Both OKT4 and OKT4A immunoprecipitate the 56kD CD4 molecule from the uninfected cells. However, in the infected cells OKT4 (but not OKT4A) immunoprecipitates CD4 as well as three molecular species of high molecular weight corresponding to viral *env* gene products that are also immunoprecipitated by immune human serum (lane 10).

Figure 3

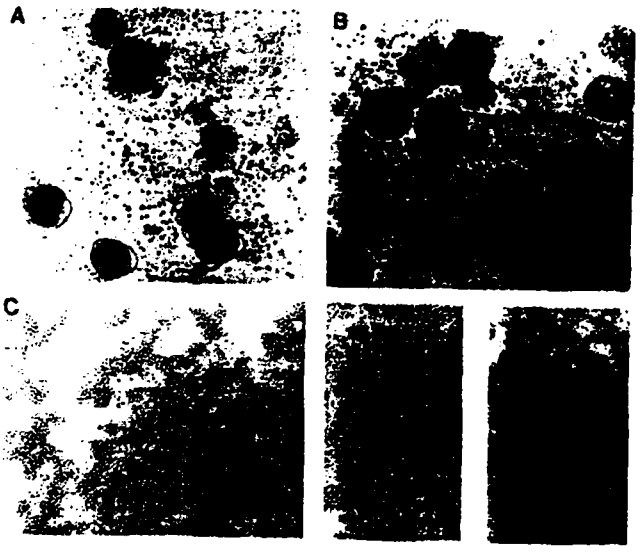

Fig. 3. Syncytium formation by HIV-2/ST, HIV-2/ROD, and HIV-1/IIIb infected cells and CD4⁺ SupT1 indicator cells. Virally-infected SupT1 cells were mixed 1:5 with uninfected SupT1 cells, incubated 24 hours, and photographed. Results were identical using Hela-T4, H9, and Hut 78 as uninfected indicator cells and when using HIV-2/ST infected H9 and Hut 78 as *env* expressing effector cells. The HIV-2/ST cell line (ST/LK001) and single cell derived clones (ST.9 and ST.24) are described in Fig. 2. (Magnification 160X.)

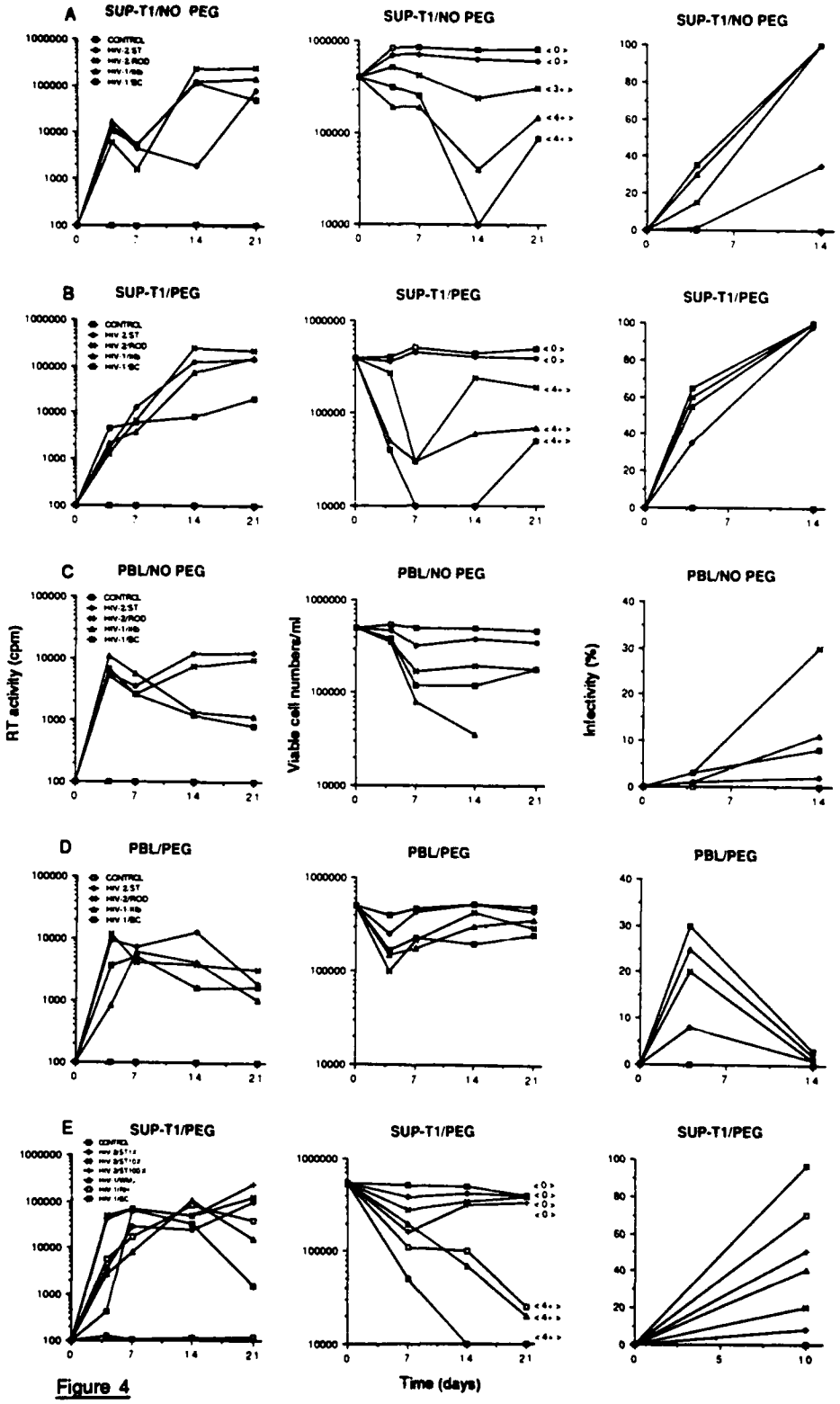

Figure 4

Fig. 4. Comparison of cytopathic/cell killing properties of HIV-2/ST with HIV-2/ROD and with HIV-1 isolates IIIb, BC, WMJ, RH. Each panel summarizes a 21 day experiment in which equivalent amounts of cell-free virus (250,000 c.p.m. RT activity) were applied to cultures of uninfected SupT1 cells (A, B) or PHA-stimulated peripheral blood lymphocytes (Panels C, D). In Panel E, 7,500 c.p.m. RT activity of all viruses were used except for HIV-2/ST 10X (75,000 c.p.m.) and HIV-2/ST 100X (750,000 c.p.m.). Shown are supernatant reverse transcriptase activities; percentages of cells infected with virus as determined by indirect immunofluorescence; and cell killing assessed by viable cell counts with trypan blue exclusion, hemocytometry, and automated cell counting. Identical culture splits were done weekly to keep the concentration of control cells between 0.5 x 10^6 and 1.0 x 10^6/ml and cell killing was expressed logarithmically as decreases in viable cell numbers. Syncytia were scored from 0 (none) to 4+ (extensive) and are shown in brackets (< >). Mock infected culture supernatants lacking virus served as controls. Ten percent (w/v) final concentration of polyethylene glycol (PEG) was used to concentrate virus for experiments shown in Panels B, D, and E.

Figure 5

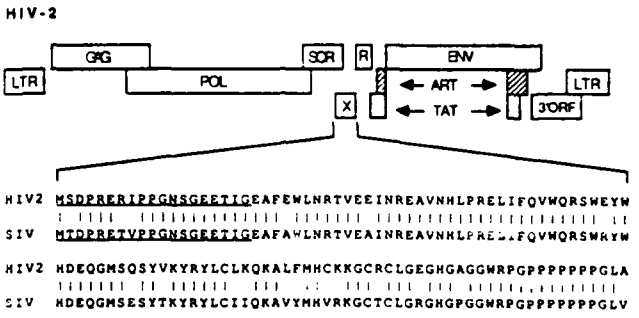

Fig. 5. Alignment of the deduced X amino acid sequence of HIV-2 and SIV_{MAC}. The genomic organization of HIV-2 is depicted as reported by Guyader, et al. (21). Shown are eight open reading frames known to encode HIV proteins as well as the novel X open reading frame. A comparison of the deduced X amino acid sequence is shown below for HIV-2 (isolate ROD, ref. 21) and SIV_{MAC} (isolate PK82, refs. 13 and 18). Vertical bars indicate sequence identity. The first 19 residues (underlined in the HIV-2 and SIV_{MAC} X sequence) represent the oligopeptides which were synthesized to raise rabbit anti-X immune sera.

Figure 6

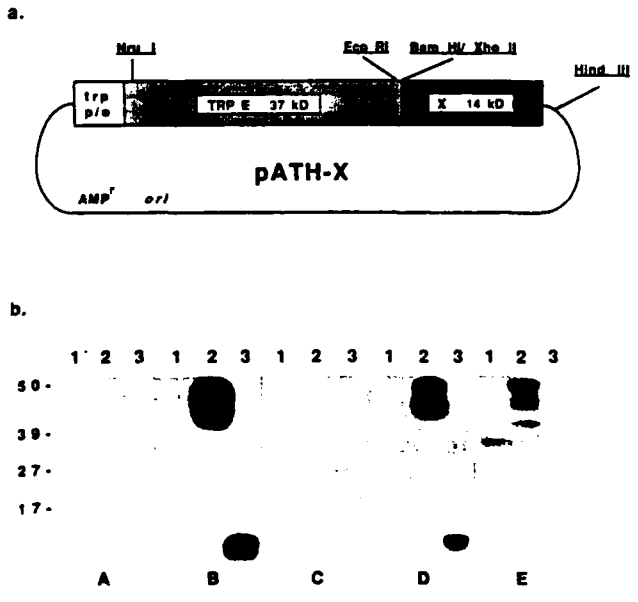

Fig. 6. Prokaryotic expression of the SIV_{MAC} X protein. (A) Construction of pATH-X. A prokaryotic expression vector, pATH, (32) was used to express the entire SIV_{MAC} X open reading frame minus the first two amino acid residues. A 422 base pair XhoII/HindIII fragment of SIV_{MAC} clone PKE102 was inserted into pATH at the BamHI and HindIII sites as depicted. This placed the SIV_{MAC} orf inframe and downstream of the bacterial Trp-E gene to allow its expression as a 50kD fusion protein. A unique NruI and EcoRI site were subsequently used to remove 95% of the Trp-E coding region, leaving only 16 TrpE derived amino acids on the 5' end of the expressed SIV_{MAC} X gene. This construct yielded a smaller fusion protein of 15kD. The position of the trp promoter/operon within the construct is indicated. (B) Western blot analysis of bacterially-expressed SIV_{MAC} X proteins. *E. coli* cell lysates transformed with non-recombinant vector pATH (lanes 1), pATH-X (lanes 2) and the TrpE deleted pATH-X construct (lanes 3) were tested by immunoblot analysis for reactivity to rabbit sera immunized with SIV_{MAC} and HIV-2 X peptides (panels B and D, respectively) as well as to preimmune rabbit sera (panels A and C). A 50kD TrpE/X and a 15kD delta TrpE/X fusion protein were recognized only by X peptide immune sera. (A smaller band of approximately 40kD represents a prematurely truncated version of the 50kD TrpE/X protein.) An antiserum raised against the bacterial TrpE gene product (panel E) was used as control to confirm the size of the 50kD TrpE/X fusion protein (lane 2) and to identify the non-recombinant 37kD TrpE protein (lane 1). This antiserum failed to detect the TrpE deleted 15kD X protein (lane 3). Antigen antibody complexes were detected using ^{125}I labelled protein A.

Figure 7

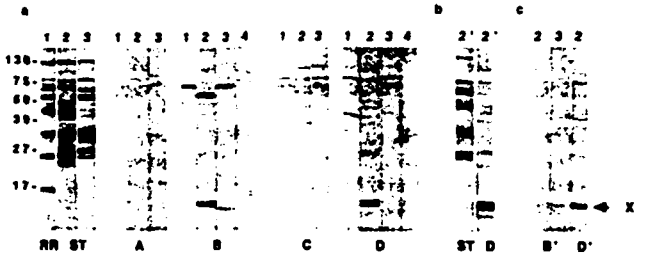

Fig. 7. Identification of the HIV-2 and SIV_{MAC} X protein. (A) Immunoblot of viral preparations probed with X peptide immune and preimmune sera. HIV-1 (isolate IIIb, lanes 1), HIV-2 (isolate ST, lanes 2), and SIV_{MAC} viral lysates (isolate PK82, lanes 3), and lysates of uninfected cell culture supernatants (lanes 4) were separated on a 12.5% polyacrylamide-sodium dodecyl sulfate gel, electrophoretically transferred to nitrocellulose, and reacted to the following sera diluted 1:100 in PBS containing 5% nonfat dry milk and 0.1% Tween 20: human anti-HIV-1 positive serum (RR); human anti-HIV-2 positive serum (ST); rabbit SIV X peptide preimmune (A) and immune serum (B); rabbit HIV-2 X peptide pre-immune (C) and immune serum (D). Bound antibody was detected by reaction with horseradish peroxidase conjugated anti-rabbit and anti-human immunoglobulins using diamianobenzidine as a substrate. (B) Immunoblots of sucrose banded, purified HIV-2 virions. HIV-2 viral supernatants (isolate ST) were clarified by low-speed centrifugation, virus was pelleted through a 20% sucrose cushion and then banded in a 20% to 60% continuous sucrose gradient. Immunoblots were prepared and identical strips containing purified HIV-2 virions as antigen (2') were reacted with an anti-HIV-2 positive human serum (ST) and the rabbit HIV-2 X peptide immune sera (D). (C) Competitive adsorption assay using bacterially expressed SIV_{MAC} X protein to adsorb anti-X antibodies. Bacterial lysates of pATH-X transformed *E. coli* immobilized on nitrocellulose were incubated with SIV_{MAC} and HIV-2 X peptide immune sera (panel B' and D', respectively). Preadsorbed sera were subsequently tested by Western blot analysis for reactivity with preparations of HIV-2 (isolate ST, lanes 2) and SIV_{MAC} (isolate PK82, lane 3).

Figure 8

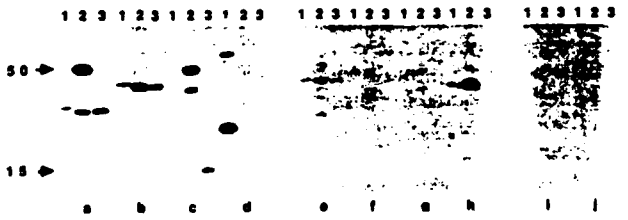

Fig. 8. Analysis of human sera for reactivity with the SIV_{MAC} X proteins expressed in *E. coli*. Cell lysates transformed with pATH vector (lanes 1), pATH-X (lanes 2) and the TrpE deleted pATH-X construct (lanes 3) were separated on a 12.5% acrylamide sodium dodecyl sulfate gel, transferred to nitrocellulose and analyzed by immunoblotting for reactivity to four HIV-2 antibody positive human sera (a-d), four HIV-1 positive human sera (e-h) and 2 normal control sera (i, j). Sera were diluted 1:50 in PBS containing 5% non-fat dry milk and 0.1% Tween 20. Arrows denote the position of the 50kD TrpE/X and the 15kD delta TrpE/X fusion proteins recognized by two of four HIV-2 positive human sera (a and c). Bound antibody was detected by ^{125}I labelled protein A.